

<https://helda.helsinki.fi>

Precise immunomodulation of the M1 to M2 macrophage transition enhances mesenchymal stem cell osteogenesis and differs by sex

Nathan, K.

2019-10

Nathan , K , Lu , L Y , Lin , T , Pajarinen , J , Jämsen , E , Huang , J-F , Romero-Lopez , M , Maruyama , M , Kohno , Y , Yao , Z & Goodman , S B 2019 , ' Precise immunomodulation of the M1 to M2 macrophage transition enhances mesenchymal stem cell osteogenesis and differs by sex ' , Bone & Joint Research , vol. 8 , no. 10 , pp. 481-488 . <https://doi.org/10.1302/2046-3758.810.BJR-2018-0231.R2>

<http://hdl.handle.net/10138/308139>

<https://doi.org/10.1302/2046-3758.810.BJR-2018-0231.R2>

unspecified

publishedVersion

Downloaded from Helda, University of Helsinki institutional repository.

This is an electronic reprint of the original article.

This reprint may differ from the original in pagination and typographic detail.

Please cite the original version.

BJR



■ BONE BIOLOGY

Precise immunomodulation of the M1 to M2 macrophage transition enhances mesenchymal stem cell osteogenesis and differs by sex

K. Nathan,
L. Y. Lu,
T. Lin,
J. Pajarinen,
E. Jämsen,
J-F. Huang,
M. Romero-Lopez,
M. Maruyama,
Y. Kohno,
Z. Yao,
S. B. Goodman

Stanford University,
Stanford, California,
United States

Objectives

Up to 10% of fractures result in undesirable outcomes, for which female sex is a risk factor. Cellular sex differences have been implicated in these different healing processes. Better understanding of the mechanisms underlying bone healing and sex differences in this process is key to improved clinical outcomes. This study utilized a macrophage–mesenchymal stem cell (MSC) coculture system to determine: 1) the precise timing of proinflammatory (M1) to anti-inflammatory (M2) macrophage transition for optimal bone formation; and 2) how such immunomodulation was affected by male *versus* female cocultures.

Methods

A primary murine macrophage–MSC coculture system was used to demonstrate the optimal transition time from M1 to M2 (polarized from M1 with interleukin (IL)-4) macrophages to maximize matrix mineralization in male and female MSCs. Outcome variables included Alizarin Red staining, alkaline phosphatase (ALP) activity, and osteocalcin protein secretion.

Results

We found that 96 hours of M1 phenotype in male cocultures allowed for maximum matrix mineralization *versus* 72 hours in female cocultures. ALP activity and osteocalcin secretion were also enhanced with the addition of IL-4 later in male *versus* female groups. The sex of the cells had a statistically significant effect on the optimal IL-4 addition time to maximize osteogenesis.

Conclusion

These results suggest that: 1) a 72- to 96-hour proinflammatory environment is critical for optimal matrix mineralization; and 2) there are immunological differences in this coculture environment due to sex. Optimizing immunomodulation during fracture healing may enhance and expedite the bone regeneration response. These findings provide insight into precise immunomodulation for enhanced bone healing that is sex-specific.

Cite this article: *Bone Joint Res* 2019;8:481–488.

Keywords: Macrophages, Mesenchymal stem cells, Coculture, Osteogenesis, Sex differences

Article focus

- Mechanisms of bone regeneration via primary macrophage–mesenchymal stem cell (MSC) interaction in an *in vitro* coculture model.
- Temporal modulation of proinflammatory (M1) to anti-inflammatory (M2) polarization to maximize MSC matrix mineralization.
- Sex differences between cells derived from male and female mice.

Key messages

- Bone mineralization at four weeks is highest with interleukin (IL)-4 addition at 96 hours in male cocultures and 72 hours in female cocultures.
- Early and late osteogenic markers were enhanced with IL-4 addition later in male cocultures compared with female cocultures.
- The sex of the cells had a statistically significant effect on the optimal time for IL-4 addition for osteogenesis.

Correspondence should be sent to
S. B. Goodman;
email: goodbone@stanford.edu

doi: 10.1302/2046-3758.810.
BJR-2018-0231.R2

Bone Joint Res 2019;8:481–488.

Strengths and limitations

- This study demonstrated the temporal effect of M1 to M2 polarization on MSC matrix mineralization in primary murine coculture system.
- This is the first study to examine and demonstrate differences in macrophage-MSC interactions based on the sex of the cells.
- Further studies are needed to explore mechanisms of the differences found between cells derived from male *versus* female mice.

Introduction

Bone fractures are among the most common injuries treated by orthopaedic surgeons, with nearly 3.9 million emergency department visits in the United States every year.¹ These fractures are estimated to cost between \$12 billion and \$18 billion in direct healthcare expenditures annually. Furthermore, indirect costs associated with bone fractures contribute billions of additional dollars. With the ageing population, these costs could double or triple in the coming decades.² Many factors contribute to the risk of fracture, including age, sex, race, and physiology, and similar characteristics are predictive of outcomes such as morbidity, death, disability, and cost to the healthcare system.³ Even with standard of care treatment, between 5% and 10% of fractures result in undesirable outcomes, such as delayed healing or nonunion.⁴⁻⁷ Such outcomes lead to complex, expensive, and often invasive alternative treatment strategies, which place undue burdens on the patient, physician, and society.⁸

In normal fracture healing, a proinflammatory cascade initiates fracture healing. Local and migrating macrophages at the site of injury phagocytose debris and release cytokines and chemokines, which promote angiogenesis, recruit MSCs, and initiate regenerative processes.⁹⁻¹¹ This proinflammatory process begins immediately after injury and peaks at 24 hours. Within a few days, the acute inflammatory reaction subsides and regenerative mechanisms start to predominate at the fracture site.¹²

Macrophages can be generally categorized into three phenotypes: undifferentiated M0, proinflammatory M1, and anti-inflammatory M2. These phenotypes exist along a spectrum and macrophages are able to transition among these phenotypes.¹³⁻¹⁵ Macrophages can be 'polarized' between these three phenotypes using various cytokines. Lipopolysaccharide (LPS) is one example of a cytokine that may be used to polarize undifferentiated M0 macrophages to the inflammatory M1 state, while interleukin (IL)-4 or IL-13 will polarize M0 or M1 macrophages to the anti-inflammatory M2 state.¹⁶⁻¹⁹

Depending on the local immune environment, macrophages are known to affect MSC growth and differentiation. M1 macrophages have been shown to inhibit human MSC (hMSC) growth, whereas M2 macrophages

support hMSC growth.²⁰ However, previous work using primary murine MSCs or MC3T3 osteoprogenitor cells demonstrated that the addition of M1 macrophages enhanced bone mineralization and osteogenesis.^{21,22} Moreover, it was shown that initiating the M1 to M2 transition at 72 hours via the addition of IL-4 resulted in enhanced matrix mineralization, alkaline phosphatase (ALP) activity, and osteocalcin secretion.²²

Interestingly, fracture healing and risk of nonunion vary with sex. In elderly populations, men are at higher risk for increased morbidity, postoperative complications, and mortality after hip fractures, while women are at higher risk for developing osteoporosis (a risk factor for fractures) and nonunions after femoral neck fractures.²³⁻²⁵ Following fractures, female rats have biomechanically compromised and radiologically delayed bone formation compared with male rats, and this effect is even greater in aged populations. These differences are, in part, due to a decreased number of MSCs in female rats.^{26,27} Moreover, male osterix-mCherry mice with induced mid-femur fractures formed larger calluses and had higher volumetric bone mineral density and bone strength than their female counterparts.²⁸ Despite the elevated bone mineral density and bone strength in male mice, the same study found that female mice had higher systemic bone formation than male mice after an induced femur fracture.²⁸ The sex of the cells has also been shown to affect the function of macrophages and MSCs, but little is known of the effect of sex on the interaction between MSCs and macrophages.²⁹⁻³²

A better understanding of the mechanisms underlying bone regeneration and the role of sex differences in this process are key to improved clinical outcomes. Few studies have examined the dynamic interplay between macrophages and MSCs in a coculture system and none have explored the temporal relationship of macrophage polarization on MSC osteogenesis.^{20,21,33,34} There is a paucity of literature on the effects of sex on the interactions between macrophages and MSCs. In the present study, we used a polarized macrophage-MSC coculture system to determine: 1) the precise timing of M1 to M2 transition for optimal bone formation; and 2) how such immunomodulation was affected by male *versus* female cocultures.

Materials and Methods

Mouse primary macrophage isolation. Primary mouse macrophages were obtained from the bone marrow of five male and five female Jackson C57BL-6J mice (Jackson Laboratory, Bar Harbor, Maine) and cells from both sexes were treated separately. All mice were eight weeks old and therefore had not reached maturity. After extraction of hind limb long bones, bone marrow was flushed into a 50 ml centrifuge tube with 5 ml of basal medium (RPMI 1640 (Thermo Fisher Scientific, Waltham, Massachusetts),

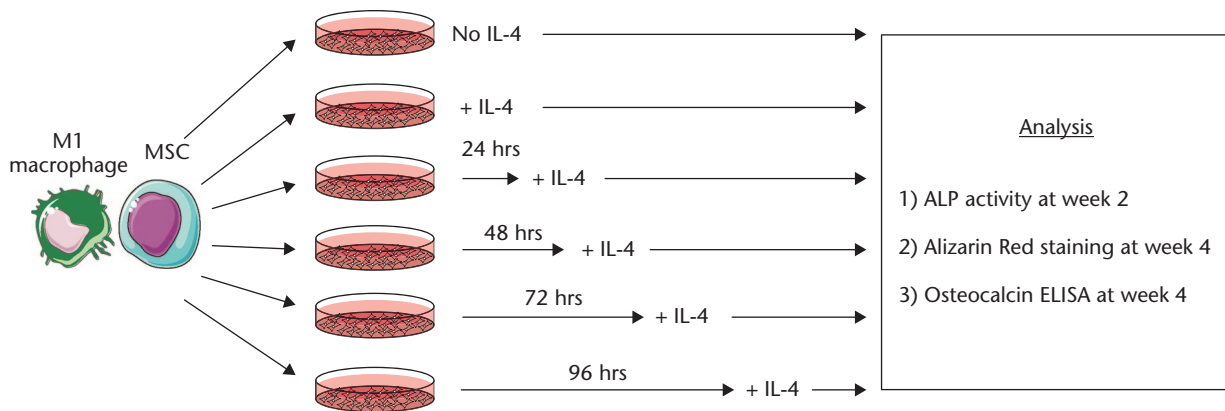


Fig. 1

Schematic demonstrating experimental methodology. Proinflammatory macrophages (M1s) were plated with mesenchymal stem cells (MSCs), at which point interleukin (IL)-4 was added immediately, after 24 hours, after 48 hours, after 72 hours, or after 96 hours. Controls included M1s cultured with MSCs with no IL-4 added, a negative control (MSCs alone in nonosteogenic growth media, not shown), and a positive control (MSCs alone in mixed osteogenic-macrophage media, not shown). Osteogenesis outcome measures included alkaline phosphatase (ALP) activity at two weeks, Alizarin Red staining at four weeks, and osteocalcin secretion at four weeks. ELISA, enzyme-linked immunosorbent assay.

10% fetal bovine serum (FBS, Thermo Fisher Scientific), 1× antibiotic-antimycotic (100 units of penicillin, 100 µg of streptomycin, and 0.25 µg of amphotericin B per milliliter, HyClone; Thermo Fisher Scientific) with a needle. A 70 µm cell strainer was used to filter cells into a separate tube. This tube was centrifuged at 400 g for ten minutes, after which cells were resuspended in 1 ml of ice-cold RBC lysis buffer (MilliporeSigma, Burlington, Massachusetts) for two minutes at 4°C; 20 ml of basal medium was then added. After centrifugation again at 400 g for ten minutes, cells were resuspended in 5 ml of augmented basal macrophage medium (RPMI 1640, 30% L929 leucocyte-conditioned medium (LCM, medium conditioned by L929 leukocytes in laboratory), 10% FBS, 1× antibiotic-antimycotic, and 10 ng/ml mouse macrophage colony-stimulating factor (mM-CSF, R&D Systems, Minneapolis, Minnesota)). Cells were counted and frozen in vials with 10% dimethyl sulfoxide (DMSO, VWR, Radnor, Pennsylvania) in serum.

Primary mouse mesenchymal stem cell isolation. Primary mouse MSCs were obtained from the bone marrow of five eight-week-old male and five eight-week-old female Jackson C57BL-6J mice (Jackson Laboratory, Bar Harbor, Maine) and cells from both sexes were treated separately. All mice were eight weeks old and therefore had not reached maturity. After extraction of hind limb long bones, bone marrow was flushed into a dish and resuspended in MSC growth medium (Minimum Essential Medium Eagle alpha (α-MEM, Thermo Fisher Scientific), 10% heat inactivated MSC qualified FBS, 1× antibiotic-antimycotic) using a needle. A 70 µm cell strainer was used to filter cells into a separate tube. This tube was centrifuged at 400 g for five minutes, after which cells were resuspended in medium and plated onto 175 cm² culture flasks. MSCs were cultured in an incubator for three to four weeks with regular medium changes. When confluent, cells

were washed with Dulbecco's phosphate-buffered saline (DPBS, Thermo Fisher Scientific) and detached by incubating in trypsin for two minutes. Detached cells were flushed with 10 ml of medium and spun at 400 g for five minutes, after which cells were resuspended and plated at a 4000 cells/cm² density. This was repeated for four to seven passages, until MSCs were counted and frozen in vials with 10% DMSO in serum.

Macrophage-MSC coculture and macrophage polarization. Frozen macrophages and MSCs were reconstituted in macrophage and MSC growth medium, respectively, and expanded to the desired cell number separately. Macrophages were polarized to M1 by 24-hour exposure to 100 ng/ml lipopolysaccharide (LPS, Sigma-Aldrich, St. Louis, Missouri) in macrophage medium, following previously established protocols.^{22,35} Polarized M1 macrophages and primary MSCs were plated concurrently in a 5:1 ratio (50 000 macrophages:10 000 MSCs) in a 24-well plate. The coculture system utilized a mixed osteogenic-macrophage medium comprised 50% osteogenic and 50% macrophage medium (44.5% α-MEM, 38.5% RPMI 1640, 10% MSC FBS, 5% LCM, 1% antibiotic-antimycotic, 1% Glutamax (Life Technologies, Carlsbad, California) supplemented with 50 µg/ml L-ascorbic acid, 0.01 M β-glycerophosphate, and 10 nM dexamethasone (all from Life Technologies). After plating of cocultures, IL-4 (20 ng/ml, R&D Systems) was added to coculture groups at 24-hour intervals (0, 24, 48, 72, and 96 hours after plating; Fig. 1). This polarization technique with LPS and IL-4 has been previously shown to produce M1 and M2 phenotypes reliably via flow cytometry, quantitative real-time polymerase chain reaction (qRT-PCR), and cytokine secretion profile.^{22,35,36}

Control groups included a negative control group (MSCs alone in MSC growth medium), a positive control group (MSCs alone in mixed osteogenic/macrophage

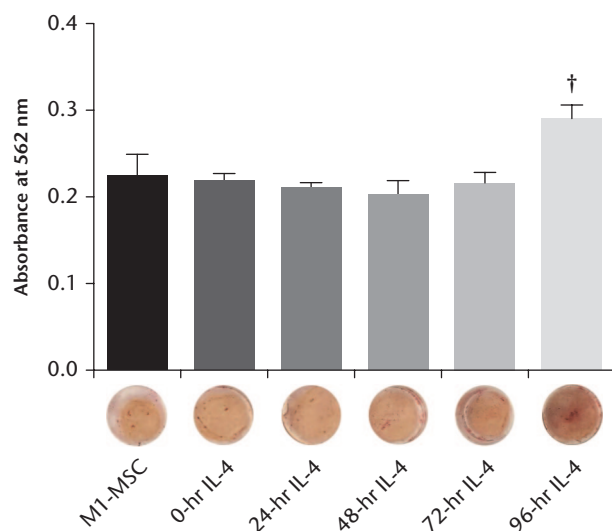


Fig. 2a

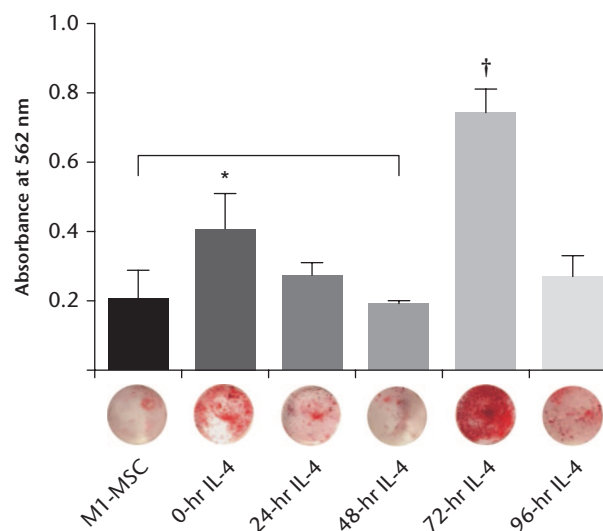


Fig. 2b

Bone matrix mineralization was measured via Alizarin Red staining at week 4. a) The male cells showed significantly increased bone mineralization after addition of interleukin (IL)-4 at 96 hours compared with all other groups ($p < 0.001$). b) In the female coculture, addition of IL-4 at 72 hours showed significantly increased Alizarin Red staining compared with all other groups ($p < 0.001$). *Statistically significant difference from groups indicated by black bar. †Statistically significant difference from all other groups. M1, proinflammatory macrophage; MSC, mesenchymal stem cell.

medium), and an M1-MSC control group without IL-4. With all three osteogenic outcome measures (Alizarin Red staining, ALP activity, and osteocalcin secretion), it was shown that the positive control groups exhibited significantly greater osteogenesis than the negative control groups, providing validation of the osteogenesis model (Supplementary Figures a, b, and c).

Detecting osteogenic markers. Alkaline phosphatase (ALP) activity was measured in cell lysates at two weeks after the beginning of cocultures using the QuantitChrom Alkaline Phosphatase Assay Kit (DALP-250, BioAssaySystems, Hayward, California). An enzyme-linked immunosorbent assay (ELISA) for osteocalcin was performed on cell supernatants collected at four weeks using the Mouse Osteocalcin ELISA Kit (Alfa Aesar, Haverhill, Massachusetts).

Alizarin Red staining. Alizarin Red staining was used to measure osteogenesis at four weeks. The cells were rinsed with DPBS followed by fixation in 2% paraformaldehyde (VWR) for ten minutes at room temperature. DPBS was used to rinse cells twice, after which 40 mM Alizarin Red solution (pH 4.1 to 4.3, Sigma-Aldrich) was used to stain cells on a rocker for 15 minutes. The cells were washed three times with deionized water on a rocker for five minutes each wash. Plates were imaged and 1 ml of 10% cetylpyridinium chloride destain solution was added to each well and placed on a rocker for one hour. Samples were transferred to a 96-well plate, and absorbance at 562 nm was read in triplicate.

Statistical analysis. In order to analyze the effect of the IL-4 addition at various timepoints on osteogenesis, a one-way analysis of variance (ANOVA) followed by Tukey's *post hoc* test was performed using GraphPad

Prism (GraphPad, San Diego, California), with $p < 0.05$ selected as the threshold for statistical significance. A two-way ANOVA was also performed to analyze the effect of the sex of the cells on osteogenesis with varying IL-4 timepoints. Bar graphs are presented as mean and standard deviation.

Results

Bone mineralization at four weeks. Alizarin Red staining of the male coculture system at four weeks (Fig. 2a) showed significantly increased matrix mineralization after addition of IL-4 at 96 hours compared with all other groups ($p < 0.001$). In the female coculture (Fig. 2b), Alizarin Red staining at four weeks showed significantly increased matrix mineralization after addition of IL-4 at 72 hours compared with all other groups ($p < 0.001$). In the female coculture, the 0-hour IL-4 group showed significantly increased matrix mineralization compared with the M1-MSC and 48-hour IL-4 groups ($p < 0.01$).

Alkaline phosphatase activity at four weeks. In the male coculture system (Fig. 3a), IL-4 added at 96 hours resulted in significantly higher ALP activity than all other groups ($p < 0.01$). Additionally, the 0-hour IL-4 group showed significantly increased ALP activity compared with the M1-MSC and 48-hour IL-4 groups ($p < 0.05$). In female cocultures (Fig. 3b), addition of IL-4 at 48 and 72 hours resulted in significantly increased ALP activity compared with IL-4 added at 0 or 24 hours ($p < 0.01$). The 96-hour IL-4 group was also found to have significantly higher ALP activity than the 24-hour IL-4 group ($p < 0.05$), but not the 0-hour IL-4 group.

Osteocalcin secretion at four weeks. In male cocultures (Fig. 4a), the addition of IL-4 at 72 and 96 hours resulted

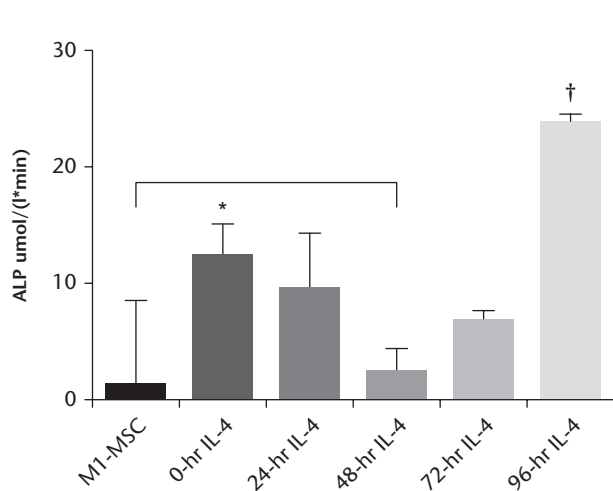


Fig. 3a

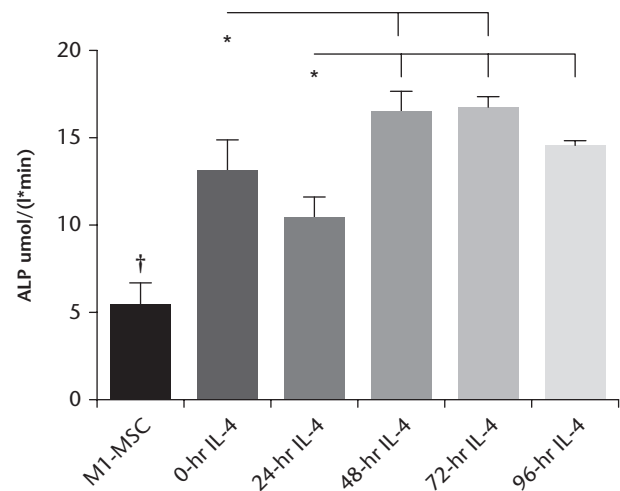


Fig. 3b

Alkaline phosphatase (ALP) activity at week 2. a) Interleukin (IL)-4 added at 96 hours to the male coculture resulted in significantly higher ALP activity than all other groups ($p < 0.01$). b) The female coculture showed that adding IL-4 at 48 and 72 hours resulted in significantly increased ALP activity compared with IL-4 added at 0 or 24 hours ($p < 0.01$). The 96-hour IL-4 group was also found to have significantly higher ALP activity than the 24-hour IL-4 group ($p < 0.05$), but not the 0-hour IL-4 group. *Statistically significant difference from groups indicated by black bars. †Statistically significant difference from all other groups. M1, proinflammatory macrophage; MSC, mesenchymal stem cell.

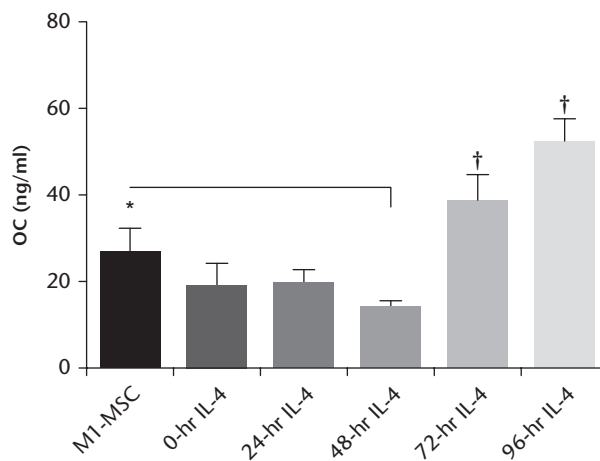


Fig. 4a

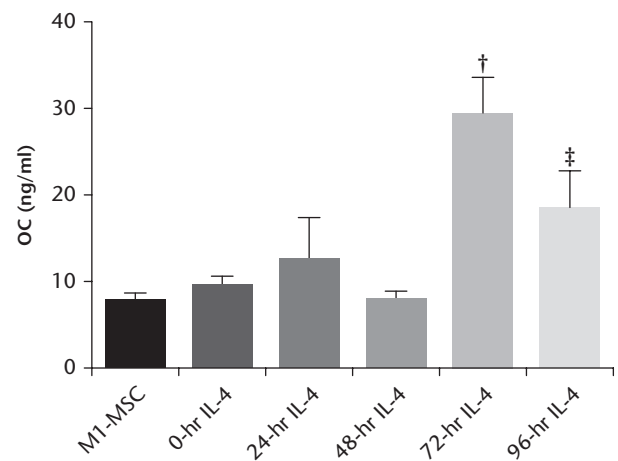


Fig. 4b

Osteocalcin secretion via enzyme-linked immunosorbent assay (ELISA) at four weeks. a) The addition of interleukin (IL)-4 at 72 and 96 hours in the male coculture resulted in significantly higher osteocalcin secretion at four weeks than all other groups ($p < 0.05$ for 72-hour IL-4; $p < 0.01$ for 96-hour IL-4). b) In the female cells, adding IL-4 at 72 hours resulted in significantly higher osteocalcin secretion than all other groups ($p < 0.01$). Addition of IL-4 at 96 hours resulted in significantly decreased osteocalcin secretion compared with the 72-hour IL-4 group ($p < 0.01$). *Statistically significant difference from groups indicated by black bars. †Statistically significant difference from all other groups. ‡Statistically significant difference from all other groups except female 24-hour IL-4. M1, proinflammatory macrophage; MSC, mesenchymal stem cell.

in significantly higher osteocalcin secretion at four weeks than all other groups ($p < 0.05$ for 72-hour IL-4; $p < 0.01$ for 96-hour IL-4). In female cocultures (Fig. 4b), the addition of IL-4 at 72 hours resulted in significantly higher osteocalcin secretion than all other groups ($p < 0.01$). Furthermore, addition of IL-4 at 96 hours resulted in significantly increased osteocalcin secretion compared with all groups except the 24-hour IL-4 group ($p < 0.05$) and significantly decreased osteocalcin secretion compared with the 72-hour IL-4 group ($p < 0.01$).

Sex of the cells had a significant effect on osteogenesis. A two-way ANOVA was performed to compare the interaction effect between sex of the cells and timing of IL-4 addition on Alizarin Red staining, ALP activity, and osteocalcin secretion. For Alizarin Red staining, the interaction effect yielded an F ratio of $F(5,36) = 39.00$ ($p < 0.001$). For ALP activity, the interaction effect yielded an F ratio of $F(5,36) = 17.48$ ($p < 0.001$) and for osteocalcin secretion, the interaction effect yielded an F ratio of $F(5,36) = 14.52$ ($p < 0.001$). This confirms that sex of the cells does

significantly affect all three osteogenesis outcome variables regarding the timing of IL-4 addition.

Discussion

The bone formation process is dependent on crosstalk between macrophages and MSCs and is characterized by an initial M1-mediated proinflammatory period, followed by an M2-mediated anti-inflammatory period.^{10,37} This study was designed to model the transition between these two vital periods by adding IL-4 to an M1-MSc coculture at various timepoints to determine the optimal time for the M1 to M2 transition.

Using male cells, adding IL-4 at 96 hours led to increased matrix mineralization, ALP activity, and osteocalcin secretion. However, using female cells, adding IL-4 earlier at 72 hours resulted in the highest levels of matrix mineralization, ALP activity, and osteocalcin secretion. These results reiterate that a finite proinflammatory period is necessary prior to transition between the M1- and M2-mediated phases in a macrophage-osteoprogenitor cell coculture system.²² Moreover, we found that this optimal time differs between cells derived from male *versus* female mice. Thus, the optimal duration of the initial inflammatory response for bone regeneration varies by sex. Of note, ALP activity in female cells was elevated but not significantly different among the 48-, 72-, and 96-hour groups, while male cells showed a marked increase in ALP activity at 96 hours compared with all other groups. However, in the setting of bone fractures, where bone mineralization is the most clinically important endpoint, the enhanced mineralization (via Alizarin Red staining) at 72 hours in the female group should be highlighted as the main finding, especially with congruent osteocalcin findings. Although ALP activity is widely used as a marker for osteogenesis, it is only actually required for the initiation of mineralization, not necessarily for the progression or as a specific indicator of final bone formation.^{38,39}

Male cells seem to benefit from a longer inflammatory response, while a slightly shorter period of inflammation is sufficient for female cells. The three- to five-day time range contains important events for bone regeneration; one study showed that there are striking differences between cancellous and cortical bone formation that occur between days 3 and 5.⁴⁰ Our study also suggests that the initial three or four days of macrophage-MSc crosstalk and macrophage immunomodulation during this time are crucial for enhanced osteogenesis four weeks later.

Variations between male and female stem cells can begin to explain these sex differences. Female stem cells have been shown to possess a greater skeletal muscle regeneration efficiency, while male stem cells have higher osteogenic potential and bone regenerative capacity.²⁹⁻³¹ However, male cells required a longer period of proinflammatory exposure than female cells to optimize

osteogenesis in our coculture system, which suggests that the addition of macrophages may contradict previously established results. One possible mechanism for these sex-linked differences is the difference in steroid receptors between males and females; these receptors are vital contributors to and mediators of stem cell proliferation and differentiation.⁴¹

There are also considerable sex-related differences with regard to inflammatory disorders in humans, most notably the higher incidence of autoimmune diseases among women.^{32,42} These differences are at least in part due to leucocyte variations; it has been shown that female mice and rats have more macrophages and lymphocytes, and female resident macrophages express higher levels of toll-like receptors (TLRs), greater phagocytosis capabilities, and enhanced bacterial killing compared with their male counterparts. However, it was concurrently shown that *in vivo* exposure of macrophages to live bacteria caused less severe sepsis and lower levels of bacteria in the blood of female mice, compared with male mice.⁴³

Additionally, several studies have shown that female-derived cells tend to exist in a lower inflammatory state than male cells.⁴⁴⁻⁴⁹ For example, a murine study investigating the acute phase of viral myocarditis demonstrated that myocardial infiltrating macrophages in male mice were predominantly of the M1 phenotype, while the corresponding cells in female mice were predominantly of the M2 phenotype.⁴⁴ Moreover, female macrophages produced a greater amount of IL-4, while male macrophages expressed higher levels of interferon-gamma (IFN- γ). Interestingly, expression of IL-13, another classical M2 cytokine, did not differ between the sexes.⁴⁴ However, others have shown that higher levels of plasma IL-6 are measured in male mice compared with female mice following exposure to LPS.⁴⁵ This suggests that the phenotypic differences between male and female macrophages may be specific to certain cytokines.

Our study has several limitations: first, this is an *in vitro* study that aims to simplify the complex physiological environment *in vivo*. Especially in the context of sex, our coculture model does not account for the systemic hormonal differences characteristic for males and females. Numerous studies have shown that sex hormones can regulate and affect cell growth/apoptosis, migration, and function of macrophages in various ways.⁵⁰⁻⁵⁴ This study's coculture model was designed and based on multiple prior studies published from our group that had previously established and validated the macrophage-MSc coculture and polarization models.^{21,22,55} However, future *in vitro* studies should incorporate appropriate sex hormones and progress to an *in vivo* model to examine the effects of sex hormones on macrophage-MSc crosstalk.

Second, though we did not directly measure the effect of IL-4 on MSCs alone, a previous study showed that IL-4 has no effect on Runx-related transcription factor 2 (RUNX2) and ALP expression in human MSCs.⁵⁶ Therefore,

the enhanced osteogenesis by addition of IL-4 is likely mediated via the crosstalk between MSCs and macrophages. Further studies should also explore timepoints later than 96 hours. As the male group maximized osteogenesis with addition of IL-4 at the final timepoint (96 hours), it is conceivable that the actual peak lies past 96 hours. Regardless, it is evident from the current data that male MSCs benefit from prolonged M1 exposure compared with female MSCs. Finally, all cells used in this study were harvested from young, immature mice. It is known that ageing causes intrinsic changes to both macrophages and MSCs.⁵⁷ Further studies should be done to investigate the effect of ageing on both macrophage-MSC crosstalk and sex differences.

In conclusion, this study found that: 1) precise immunomodulation of the M1 to M2 transition is essential for enhanced osteogenesis of MSCs; and 2) this optimal inflammatory period differs between male and female coculture systems. Optimizing immunomodulation during fracture healing may enhance and expedite the bone regeneration response. Moreover, these results suggest that there are sex differences that are associated with macrophage-MSC crosstalk in osteogenesis. These differences at the cellular level may, in part, explain differences in bone healing clinically. As inflammation is essential for all regenerative processes, our study may have broader implications for regeneration in other organ systems and the potential differences that may exist due to sex.

Supplementary material



Data for Alizarin Red staining at four weeks, alkaline phosphatase activity at two weeks, and osteocalcin secretion at two weeks for mesenchymal stem cell (MSC) control groups (male or female MSCs cultured alone in osteogenic media, with no macrophages or interleukin 4 added). With all three osteogenic outcome measures, it was shown that the positive control groups exhibited significantly greater osteogenesis than the negative control groups, providing validation of the osteogenesis model.

References

- No authors listed.** National Hospital Ambulatory Medical Care Survey: 2013 Emergency Department Summary Tables. Centers for Disease Control and Prevention. 2013. https://www.cdc.gov/nchs/data/ahcd/nhamcs_emergency/2013_ed_web_tables.pdf (date last accessed 19 September 2019).
- No authors listed.** The burden of bone disease. In *Bone Health and Osteoporosis: A Report of the Surgeon General*. Rockville, Maryland: United States Department of Health and Human Services. 2004:88-108.
- Pressley JC, Kendig TD, Frencher SK, et al.** Epidemiology of bone fracture across the age span in blacks and whites. *J Trauma* 2011;71(Suppl 2):S541-S548.
- Kaempffe FA, Bone LB, Border JR.** Open reduction and internal fixation of acetabular fractures: heterotopic ossification and other complications of treatment. *J Orthop Trauma* 1991;5:439-445.
- Bone L, Johnson K, Weigett J, Scheinberg R.** Early versus delayed stabilization of femoral fractures. A prospective randomized study. *Orthop Trauma Dir* 2006;4:29-33.
- Tzioupis C, Giannoudis PV.** Prevalence of long-bone non-unions. *Injury* 2007;38(Suppl 2):S3-S9.
- Calori GM, Albisetti W, Agus A, Iori S, Tagliabue L.** Risk factors contributing to fracture non-unions. *Injury* 2007;38(Suppl 2):S11-S18.
- Einhorn TA, Lane JM.** Significant advances have been made in the way surgeons treat fractures. *Clin Orthop Relat Res* 1998;355(Suppl):S2-S3.
- Andrew JG, Andrew SM, Freemont AJ, Marsh DR.** Inflammatory cells in normal human fracture healing. *Acta Orthop Scand* 1994;65:462-466.
- Claes L, Recknagel S, Ignatius A.** Fracture healing under healthy and inflammatory conditions. *Nat Rev Rheumatol* 2012;8:133-143.
- Chung R, Cool JC, Scherer MA, Foster BK, Xian CJ.** Roles of neutrophil-mediated inflammatory response in the bony repair of injured growth plate cartilage in young rats. *J Leukoc Biol* 2006;80:1272-1280.
- McKibbin B.** The biology of fracture healing in long bones. *J Bone Joint Surg [Br]* 1978;60-B:150-162.
- Mosser DM, Edwards JP.** Exploring the full spectrum of macrophage activation. *Nat Rev Immunol* 2008;8:958-969.
- Italiani P, Boraschi D.** From monocytes to M1/M2 macrophages: phenotypical vs. functional differentiation. *Front Immunol* 2014;5:514.
- Martinez FO, Sica A, Mantovani A, Locati M.** Macrophage activation and polarization. *Front Biosci* 2008;13:453-461.
- Adams DO.** Molecular interactions in macrophage activation. *Immunol Today* 1989;10:33-35.
- Gordon S.** Alternative activation of macrophages. *Nat Rev Immunol* 2003;3:23-35.
- Mantovani A, Biswas SK, Galdiero MR, Sica A, Locati M.** Macrophage plasticity and polarization in tissue repair and remodelling. *J Pathol* 2013;229:176-185.
- Loi F, Córdova LA, Pajarinen J, et al.** Inflammation, fracture and bone repair. *Bone* 2016;86:119-130.
- Freytes DO, Kang JW, Marcos-Campos I, Vunjak-Novakovic G.** Macrophages modulate the viability and growth of human mesenchymal stem cells. *J Cell Biochem* 2013;114:220-229.
- Lu LY, Loi F, Nathan K, et al.** Pro-inflammatory M1 macrophages promote Osteogenesis by mesenchymal stem cells via the COX-2-prostaglandin E2 pathway. *J Orthop Res* 2017;35:2378-2385.
- Loi F, Córdova LA, Zhang R, et al.** The effects of immunomodulation by macrophage subsets on osteogenesis in vitro. *Stem Cell Res Ther* 2016;7:15.
- Poole KES, Compston JE.** Osteoporosis and its management. *BMJ* 2006;333:1251-1256.
- Parker MJ, Raghavan R, Gurusamy K.** Incidence of fracture-healing complications after femoral neck fractures. *Clin Orthop Relat Res* 2007;458:175-179.
- Endo Y, Aharonoff GB, Zuckerman JD, Egol KA, Koval KJ.** Gender differences in patients with hip fracture: a greater risk of morbidity and mortality in men. *J Orthop Trauma* 2005;19:29-35.
- Strube P, Mehta M, Baerenwaldt A, et al.** Sex-specific compromised bone healing in female rats might be associated with a decrease in mesenchymal stem cell quantity. *Bone* 2009;45:1065-1072.
- Muschler GF, Nitto H, Boehm CA, Easley KA.** Age- and gender-related changes in the cellularity of human bone marrow and the prevalence of osteoblastic progenitors. *J Orthop Res* 2001;19:117-125.
- Yao W, Lay YE, Kot A, et al.** Improved mobilization of exogenous mesenchymal stem cells to bone for fracture healing and sex difference. *Stem Cells* 2016;34:2587-2600.
- Deasy BM, Lu A, Tebbets JC, et al.** A role for cell sex in stem cell-mediated skeletal muscle regeneration: female cells have higher muscle regeneration efficiency. *J Cell Biol* 2007;177:73-86.
- Corsi KA, Pollett JB, Phillippi JA, et al.** Osteogenic potential of postnatal skeletal muscle-derived stem cells is influenced by donor sex. *J Bone Miner Res* 2007;22:1592-1602.
- Aksu AE, Rubin JP, Dudas JR, Marra KG.** Role of gender and anatomical region on induction of osteogenic differentiation of human adipose-derived stem cells. *Ann Plast Surg* 2008;60:306-322.
- Klein SL, Flanagan KL.** Sex differences in immune responses. *Nat Rev Immunol* 2016;16:626-638.
- Guihard P, Danger Y, Brounais B, et al.** Induction of osteogenesis in mesenchymal stem cells by activated monocytes/macrophages depends on oncostatin M signaling. *Stem Cells* 2012;30:762-772.
- Nicolaidou V, Wong MM, Redpath AN, et al.** Monocytes induce STAT3 activation in human mesenchymal stem cells to promote osteoblast formation. *PLoS One* 2012;7:e39871.
- Rao AJ, Gibon E, Ma T, et al.** Revision joint replacement, wear particles, and macrophage polarization. *Acta Biomater* 2012;8:2815-2823.
- Pajarinen J, Tamaki Y, Antonios JK, et al.** Modulation of mouse macrophage polarization in vitro using IL-4 delivery by osmotic pumps. *J Biomed Mater Res A* 2015;103:1339-1345.

37. Pajarinen J, Lin T, Gibon E, et al. Mesenchymal stem cell-macrophage crosstalk and bone healing. *Biomaterials* 2019;196:80-89.
38. Miao D, Scutt A. Histochemical localization of alkaline phosphatase activity in decalcified bone and cartilage. *J Histochem Cytochem* 2002;50:333-340.
39. Bellows CG, Aubin JE, Heersche JN. Initiation and progression of mineralization of bone nodules formed in vitro: the role of alkaline phosphatase and organic phosphate. *Bone Miner* 1991;14:27-40.
40. Tötting L, Sandberg O, Bernhardsson M, Ernerudh J, Aspenberg P. Different composition of leucocytes in cortical and cancellous bone healing in a mouse model. *Bone Joint Res* 2019;7:620-628.
41. Hong L, Sultana H, Paulius K, Zhang G. Steroid regulation of proliferation and osteogenic differentiation of bone marrow stromal cells: a gender difference. *J Steroid Biochem Mol Biol* 2009;114:180-185.
42. Whitacre CC. Sex differences in autoimmune disease. *Nat Immunol* 2001;2:777-780.
43. Scotland RS, Stables MJ, Madalli S, Watson P, Gilroy DW. Sex differences in resident immune cell phenotype underlie more efficient acute inflammatory responses in female mice. *Blood* 2011;118:5918-5927.
44. Li K, Xu W, Guo Q, et al. Differential macrophage polarization in male and female BALB/c mice infected with coxsackievirus B3 defines susceptibility to viral myocarditis. *Circ Res* 2009;105:353-364.
45. Everhardt Queen A, Moerdyk-Schauwecker M, McKee LM, Leamy LJ, Huet YM. Differential expression of inflammatory cytokines and stress genes in male and female mice in response to a lipopolysaccharide challenge. *PLoS One* 2016;11:e0152289.
46. Naor R, Domankevich V, Shemer S, et al. Metastatic-promoting effects of LPS: sexual dimorphism and mediation by catecholamines and prostaglandins. *Brain Behav Immun* 2009;23:611-621.
47. Moxley G, Posthuma D, Carlson P, et al. Sexual dimorphism in innate immunity. *Arthritis Rheum* 2002;46:250-258.
48. Imahara SD, Jelacic S, Junker CE, O'Keefe GE. The influence of gender on human innate immunity. *Surgery* 2005;138:275-282.
49. Marriott I, Bost KL, Huet-Hudson YM. Sexual dimorphism in expression of receptors for bacterial lipopolysaccharides in murine macrophages: a possible mechanism for gender-based differences in endotoxin shock susceptibility. *J Reprod Immunol* 2006;71:12-27.
50. Cutolo M, Capellino S, Montagna P, et al. Sex hormone modulation of cell growth and apoptosis of the human monocytic/macrophage cell line. *Arthritis Res Ther* 2005;7:R1124-R1132.
51. Houdeau E, Moriez R, Leveque M, et al. Sex steroid regulation of macrophage migration inhibitory factor in normal and inflamed colon in the female rat. *Gastroenterology* 2007;132:982-993.
52. Miller L, Hunt JS. Sex steroid hormones and macrophage function. *Life Sci* 1996;59:1-14.
53. Knöferl MW, Angele MK, Diodato MD, et al. Female sex hormones regulate macrophage function after trauma-hemorrhage and prevent increased death rate from subsequent sepsis. *Ann Surg* 2002;235:105-112.
54. Wichmann MW, Ayala A, Chaudry IH. Male sex steroids are responsible for depressing macrophage immune function after trauma-hemorrhage. *Am J Physiol* 1997;273:C1335-C1340.
55. Córdova LA, Loi F, Lin TH, et al. CCL2, CCL5, and IGF-1 participate in the immunomodulation of osteogenesis during M1/M2 transition in vitro. *J Biomed Mater Res A* 2017;105:3069-3076.
56. Omar OM, Granéli C, Ekström K, et al. The stimulation of an osteogenic response by classical monocyte activation. *Biomaterials* 2011;32:8190-8204.
57. Gibon E, Lu L, Goodman SB. Aging, inflammation, stem cells, and bone healing. *Stem Cell Res Ther* 2016;7:44.

Author information

- K. Nathan, BS, Medical Student,
- L. Y. Lu, MD, Resident Physician,
- T. Lin, DVM, PhD, Post-doctoral Fellow,
- J. Pajarinen, MD, PhD, Post-doctoral Fellow,
- J-F. Huang, MD, Visiting Scholar,
- M. Romero-Lopez, PhD, Post-doctoral Fellow,
- M. Maruyama, MD, Visiting Scholar,
- Y. Kohno, MD, Visiting Scholar,
- Z. Yao, PhD, Lab Manager, Department of Orthopaedic Surgery, Stanford University, Stanford, California, USA.
- E. Jämsen, MD, PhD Student, Department of Medicine, University of Helsinki, and Helsinki University Hospital, Helsinki, Finland.
- S. B. Goodman, MD, PhD, Professor of Orthopaedic Surgery, Principal Investigator, Department of Orthopaedic Surgery, Stanford University, Stanford, California, USA; Department of Bioengineering, Stanford University, Stanford, California, USA.

Author contributions

- K. Nathan: Designed the study, Executed the experiments, Wrote the manuscript.
- L. Y. Lu: Assisted with designing the study and executing the experiments, Wrote the manuscript.
- T. Lin: Assisted with designing the study and executing the experiments, Wrote the manuscript.
- J. Pajarinen: Assisted with designing the study and executing the experiments, Wrote the manuscript.
- E. Jämsen: Assisted with designing the study and executing the experiments, Wrote the manuscript.
- J-F. Huang: Assisted with designing the study and executing the experiments, Wrote the manuscript.
- M. Romero-Lopez: Assisted with designing the study and executing the experiments, Wrote the manuscript.
- M. Maruyama: Assisted with designing the study and executing the experiments, Wrote the manuscript.
- Y. Kohno: Assisted with designing the study and executing the experiments, Wrote the manuscript.
- Z. Yao: Provided training for lab members, Managed logistics and finances of lab operations, Assisted with designing the study and executing the experiments, Wrote the manuscript.
- S. B. Goodman: Principal investigator for project, Provided funding, advising, and mentorship, Assisted with designing the study and executing the experiments, Wrote the manuscript.

Funding statement

- The authors report grants from the National Institutes of Health (NIH) related to this study (R01AR055650, R01AR063717, and 1UG3TR002136). The work was also supported by the Ellenburg Chair in Surgery and the Stanford University Medical Scholars Research Grant. E. Jämsen reports grants from the Finnish Cultural Foundation, Emil Aaltonen Foundation, and Otto A. Malm Foundation related to this study.
- No benefits in any form have been received or will be received from a commercial party related directly or indirectly to the subject of this article.

© 2019 Author(s) et al. This is an open-access article distributed under the terms of the Creative Commons Attribution licence (CC-BY-NC), which permits unrestricted use, distribution, and reproduction in any medium, but not for commercial gain, provided the original author and source are credited.